

Reduced Skin Barrier Function Parallels Abnormal Stratum Corneum Lipid Organization in Patients with Lamellar Ichthyosis

Adriana P.M. Lavrijsen, Joke A. Bouwstra,* Gert S. Gooris,* Arij Weerheim, Harry E. Boddé,* and Maria Ponc

Department of Dermatology, University Hospital Leiden, and *Leiden/Amsterdam Center for Drug Research, University of Leiden, Leiden, The Netherlands

Most patients with autosomal recessive lamellar ichthyosis are known to have markedly impaired skin barrier function. We hypothesize that this may be due to imperfections in the composition and fine structure of the intercellular stratum corneum lipids. The aim of the present study was to test this hypothesis. To characterize the barrier properties in three female patients with lamellar ichthyosis, the following parameters were used and compared with those of healthy volunteers: transepidermal water loss, stratum corneum lipid profiles after topical acetone/ether extraction on the flexure side of the forearm, and small-angle x-ray diffraction. The extracted lipids were separated using high performance thin-layer chromatography and quantified, and the ceramide profile was determined. Small-angle x-ray diffraction was used to obtain information on the molecular structure and organization of the intercellular lipid

domains of stratum corneum using stratum corneum scales collected by scraping. Transepidermal water loss was significantly increased in all three patients. Lipid analysis showed significant differences in the relative amounts of ceramide fractions 2-3a-3b-4-5, free fatty acid-ceramide ratio, and free fatty acid-cholesterol ratio. Small-angle x-ray diffraction showed smaller repeated distances of lipid bilayers in stratum corneum samples of the patients compared with the healthy volunteers. An additional diffraction peak was found in the patients compared with the healthy volunteers, which can be ascribed to crystalline cholesterol. These data suggest that there might be a relation between the impaired barrier function and stratum corneum lipid structural and composition changes. Key words: ceramides/transepidermal water loss/small-angle x-ray diffraction/diseased skin. *J Invest Dermatol* 105:619-624, 1995

Patients with lamellar ichthyosis belong to a heterogeneous group, of which most patients are known to have markedly impaired skin barrier function [1-3]. This impaired barrier function implies a risk of acute intoxications from severely increased skin permeability. In children, this is reinforced by a greater cutaneous surface area-to-weight ratio in comparison with adults [4,5]. Intoxication with salicylic acid [6] and lindane [7] has been described in children with lamellar ichthyosis. Furthermore, high plasma urea concentrations caused by topical urea therapy have been described in two babies with lamellar ichthyosis [8]. As a result of the impaired barrier, even hypernatremic dehydration and hypothermia have been described in neonates with severe congenital ichthyosis [9,10]. Some of these neonates were preterm, and because the epidermis of preterm infants is not fully developed, an additional factor can contribute to

the barrier impairment [11-13]. Several ultrastructural studies have suggested an underlying disorder of lipid metabolism in lamellar ichthyosis [14-18].

It is generally accepted that the intercellular stratum corneum lipids constitute the predominant penetration barrier for a great variety of substances. There is increasing evidence that the stratum corneum owes its barrier properties to its intercellular lipid composition, as was reviewed by Elias and Menon [19] and Schurer and Elias [20]. In this respect, the presence of ceramides seems to be important [21].

The aim of the present study was to gain insight into the relation between the structure and the function of the barrier under pathologic conditions. The potential relation between the skin barrier function on the one hand, and the lipid composition and organization on the other hand, was investigated by comparing ichthyotic skin with that of healthy volunteers. The following *in vivo* approach was used in a study of three patients with autosomal recessive ichthyosis, also called lamellar ichthyosis, as compared with healthy volunteers. First, the barrier capacity was evaluated by transepidermal water loss, then stratum corneum lipid composition was determined by high-performance thin-layer chromatography (HPTLC) of topical extracts, and finally stratum corneum lipid organization was studied by small-angle x-ray diffraction (SAXD) of stratum corneum scrapings.

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Reprint requests to: Dr. A.P.M. Lavrijsen, Department of Dermatology, University Hospital, Rijnsburgerweg 10, 2333 AA Leiden, The Netherlands.

Abbreviations: HPTLC, high-performance thin-layer chromatography; SAXD, small-angle x-ray diffraction.

MATERIALS AND METHODS

Patients and Healthy Volunteers Three female patients with autosomal recessive lamellar ichthyosis aged 25 (patient A), 33 (patient B), and 37 (patient C) years and six volunteers (five female, one male), aged 24–50 years (healthy volunteers 1–6), participated in the study. Patients A and C are sisters.

Patient A was born without features of a collodion baby. Her present features were generalized scaling, with minimal to no erythroderma, involving all flexures, large plate-like dark brown hyperkeratosis on the extensor side of the legs, and a mild palmar-plantar keratoderma. There was involvement of the face with locally dark scales (forehead, nasolabial folds) and a slight ectropion. This clinical picture fits in the phenotype of nonerythrodermic lamellar ichthyosis. The clinical picture of patient C differed considerably from that of her sister. Her skin was more severely involved and more erythrodermic. She was born as a collodion baby, and as a neonate she was admitted to the hospital because of salicylate intoxication. Her skin showed generalized fine scaling with mild to moderate erythema involving all flexures, and larger plate-like scales on the legs. There was involvement of the face with mild ectropion and a mild palmar-plantar keratoderma. She had a mild alopecia. Her clinical picture fits in the phenotype of erythrodermic lamellar ichthyosis, also called nonbullous congenital ichthyosiform erythroderma. Patient B was born as a collodion baby. Her present features were generalized fine white scales with mild erythema, larger plate-like scales on the leg, and a mild palmar-plantar keratoderma. She had extended alopecia and a slight ectropion. As a child, her skin showed large dark and plate-like scales on the trunk, on the extremities, and in the flexures.

Topical lipid extraction was performed 1 month after oral treatment with etretinate was discontinued. The use of topical treatment or emollients on the test arm was discontinued in patients 1 month and in healthy volunteers 1 week before the experiment.

Small-angle x-ray diffraction (SAXD) was performed on stratum corneum of all three patients during a period when the patients were treated with acitretin in a daily dosage between 25 mg and 35 mg. The patients and healthy volunteers discontinued the use of topical treatment or emollients on the test arm 1 week before the experiment.

Transepidermal Water Loss (TEWL) TEWL was measured on the flexure side of the forearm in patients A, B, and C and in healthy volunteers 1–6 directly before and after scraping of the stratum corneum. TEWL expressed in g/m²/h was measured using an evaporimeter (EPI; ServoMed, Stockholm, Sweden) as described earlier [22]. We performed the measurements in a large open-top box situated in a temperature- and humidity-controlled room at a room temperature between 20.6°C and 21.4°C and at an ambient air humidity between 34% and 38%.

Lipid Collection and Analysis A noninvasive topical lipid extraction procedure was performed in the three patients and in healthy volunteers 1, 4, 5, and 6 as described in detail in a recent paper [23]. Briefly, a metal cylinder with a cross-sectional area of 7 cm², precleaned in a chloroform-methanol mixture and having a simple flat edge without any lining material, was held manually on the flexure side of the forearm throughout the extraction procedure, applying a manual force just enough to prevent lateral leakage. The procedure consisted of two subsequent extraction steps: first, a 5-min application of a 10-ml acetone/diethylether (1:1) mixture; second, a subsequent extraction for 25 min executed at the same site by applying the same amount of the same solvent. To remove scale contamination, the extract was centrifuged (800 × g). Only the second fraction was used for further analysis; the first extract was used to remove sebaceous and exogenous lipids from the stratum corneum. The supernatants were evaporated to dryness in glass tubes under a stream of nitrogen, whereupon the residues were dissolved in 1 ml chloroform/methanol (9:1) and stored at –20°C until use.

Separation of extracted lipids was performed by means of one-dimensional high-performance thin-layer chromatography (HPTLC) [24]. Lipids were fractionated using the following development system: 5–50 µg of the total lipid extract was applied to HPTLC plates and developed sequentially from the bottom edge of the plate using 1) 30 mm chloroform, 2) 10 mm chloroform-acetone-methanol (76:8:16), 3) 60 mm chloroform-hexyl acetate-acetone-methanol (86:1:10:4), 4) 20 mm chloroform-acetone-methanol (76:4:20), 5) 65 mm chloroform-diethyl ether-hexyl acetate-ethyl acetate-acetone-methanol (72:4:1:16:4), and 6) 90 mm hexane-diethyl ether-ethyl acetate (78:18:4).

For identification and quantification of lipids, the following standards were used: phosphatidylethanolamine (Sigma; P-7523); ceramides, type III (Sigma; C-2137); ceramides, type IV (Sigma; C-2512); cerebroside, type II (Sigma; C-1516); lipid standards containing oleic acid, cholesterol, triolein, and cholesterol oleate (Sigma; 178-4); cholesterol sulfate (Sigma; C-9523),

1,2-diolelin and 1,3-diolelin (Sigma; D-8894); and squalene (Sigma; S-3626). After lipid separation, the plates were charred and scanned using a Densitometer CD 60 (DESAGA) [25].

Isolation of Stratum Corneum for SAXD Stratum corneum from the three patients and from three healthy volunteers (1, 2, and 3) was collected by repeatedly scraping the skin. Scraping was performed with a single-edge razor blade (GEM Scientific) on a skin area of approximately 3 × 8 cm on the flexure side of the forearm. Each skin area was subjected to about 20 consecutive scrapings. The stratum corneum samples were dried and stored over silica gel. Twenty-four hours before measurements, the samples were equilibrated over 27% NaBr, which resulted in approximately 20% hydration. Direct scraping was found to be superior to either stripping or biopsies.

SAXD All measurements were carried out at the Synchrotron Radiation Source at Daresbury Laboratories using station 8.2, which was built as part of an NWO/SERC agreement. The scattering intensities are plotted as a function of vector Q . Q is defined as $4\pi\sin\theta/\lambda$, in which θ and λ are the scattering angle and the wavelength, respectively. The wavelength at the sample position is 0.15 nm. The exposure time for all measurements was 15 min. The position of the diffraction peak is directly related to the repeating units in the structure. In a lamellar phase, the relation is $Q_n = 2n\pi/d$, in which n is the order of the diffraction peak located at Q_n and d is the corresponding spacing. A more detailed description of the equipment is given elsewhere [26].

In previous studies, it has been shown that 15 min of exposure of stratum corneum samples to x-ray did not result in changes in the scattering curve of the tissue. From this it was concluded that no detectable damage of the tissue structure occurred [26].

In the first series of experiments, the scattering curves of stratum corneum of three healthy volunteers were measured as a function of temperature varying between 25°C and 120°C. In a second series of experiments, the scattering curves of stratum corneum originating from three patients with autosomal recessive lamellar ichthyosis were measured.

Statistics The level of significance of the difference was calculated by an unpaired t test as well as a nonparametric Mann-Whitney test.

RESULTS

Elevated TEWL Baseline TEWL values in patients A, B, and C were 13.5, 15.0, and 11.7 g/m²/h, respectively (mean 13.2, standard error 1.0 with a 95% confidence interval of the mean 9.1–17.4). The mean TEWL value in the six healthy volunteers was 5.1 g/m²/h (standard error 0.3 with 95% confidence interval 4.3–6.0). The baseline TEWL values found in the patients were statistically significantly higher (unpaired t test, $p = 0.0001$) than those found in the healthy volunteers. After scraping, the TEWL values in the three patients became, respectively, 76.1, 58.0, and 81.0 g/m²/h, and in the healthy volunteers (1, 2, 3) who participated in the SAXD measurements, 23.2, 64.6, and 25.0 g/m²/h, respectively.

Abnormal Stratum Corneum Lipid Composition By analyzing the ceramide fraction by TLC, eight ceramide fractions could be recovered. These ceramide fractions were grouped into six fractions that correspond closely in terms of mobility (same R_f value) on TLC to pig epidermal ceramides [21,23]. The relative amounts of ceramide fractions 2, 3a, 3b, 4, and 5 found in patients were significantly different from those found in healthy volunteers, as shown in Fig 1. Furthermore, when comparing the ratios between the amounts corresponding to various classes of lipids using a nonparametric Mann-Whitney test, significant differences were found between patients and healthy volunteers in the ratios of free fatty acids to cholesterol and free fatty acids to ceramides, whereas the ceramides-cholesterol ratio was not different (Table I). An unpaired t test yielded similar results.

A more detailed inspection of the thin-layer chromatogram developed using the "lanosterol/lathosterol" developing system [25] revealed the presence of an unknown component with an R_f value between that of cholesterol and diglycerides (Fig 2). This unknown component was only found in the extracts of the patients. When the emollients used by the patients were extracted by chloroform-methanol and the extracted components were separated by TLC, it turned out that a major component of the emollients, cetyl alcohol, showed the same R_f value as that of the

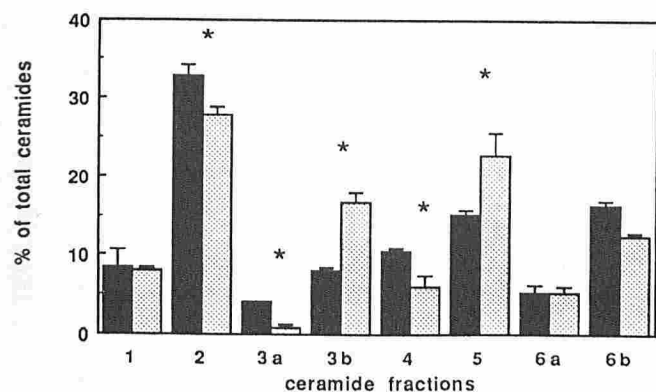


Figure 1. Ceramide profile in patients with lamellar ichthyosis and healthy volunteers. Lipids collected by topical acetone/diethylether extraction were separated by HPTLC. Diagram represents mean ceramide percentages found in three patients with lamellar ichthyosis (solid bars) and four healthy volunteers (numbers 1, 4, 5, 6) (shaded bars). Error bars, SEM. * $p < 0.05$.

unknown component present in the topical extracts of the patients. This lipid fraction could be detected in both the 5-min and the 25-min extracts (Fig 2) even though the patients had not used local treatment during 1 whole month before the lipid extraction.

Abnormal SAXD Scattering Curves The scattering curves of stratum corneum obtained by scraping from the healthy volunteers (healthy volunteers 1 and 2) are shown in Fig 3a. The scattering curves are characterized by a high intensity at small Q values (Q less than $0.6/\text{nm}$) and by a single diffraction peak at $Q = 0.98/\text{nm}$. At higher Q values, no diffraction peaks can be detected on the scattering curve. Assuming that the diffraction peak at $Q = 0.98/\text{nm}$ is of first order and based on a lamellar phase, the repeat distance of the structure is 6.4 nm .

In Fig 3b, the scattering curves are shown for stratum corneum from one volunteer measured as a function of temperature. Between 25°C and 45°C , the scattering curve did not change significantly, but at 60°C only a shoulder at the descending scattering curve remained. It seems that between 45°C and 60°C , a disordering of the lamellar stacks occurred.

In Fig 4a, the scattering curves of stratum corneum obtained by scraping from the three patients with lamellar ichthyosis are plotted and compared with that of a healthy volunteer. The main single diffraction peak of the patients is located at a significantly higher Q value than was the case for the healthy volunteers, namely $1.17/\text{nm}$, $1.18/\text{nm}$, and $1.06/\text{nm}$, corresponding to spacings of 5.35 , 5.38 , and 5.90 nm , respectively. In all three patients, a diffraction peak is found at $Q = 1.87/\text{nm}$ ($d = 3.35 \text{ nm}$), probably because of

Table I. Abnormal Stratum Corneum Lipid Composition in Patients with Lamellar Ichthyosis^a

Lipid Ratio	Patients (n = 3)	Healthy Volunteers (n = 4)	p Value, Mann-Whitney Test
FFA/sterol	0.181 (0.176–0.192)	0.834 (0.297–0.899)	0.03
FFA/CER	0.093 (0.086–0.167)	0.311 (0.227–0.405)	0.03
CER/sterol	1.884 (1.15–2.10)	2.343 (1.30–2.98)	0.16

^a Lipids collected by topical acetone/diethylether extraction were separated by HPTLC. Data are presented as median (range).

the presence of polycrystalline cholesterol. Noteworthy in patient C is the presence of an additional diffraction peak at $Q = 0.82/\text{nm}$ ($d = 7.66 \text{ nm}$). Furthermore, in patient B an additional diffraction peak at $Q = 3.15$ with a repeat distance of 1.99 nm is observed. When measurements were carried out at stepwise increasing temperatures, this peak did not disappear between 45°C and 60°C and was still present, but decreased in intensity, at 75°C (indicating a crystalline structure).

Figure 4b shows the scattering curves of the stratum corneum of patient C measured at various temperatures. As observed in the samples taken from the healthy volunteers, the main diffraction peak disappeared between 45°C and 60°C . The additional diffraction peak at $Q = 0.82/\text{nm}$ observed in patient C disappeared between 25°C and 45°C , hence the diffraction peak at $Q = 0.82/\text{nm}$ is due to a structure different from the one found at $Q = 1.17/\text{nm}$. The cholesterol peaks disappeared between 60°C and 75°C .

DISCUSSION

In most studies designed to examine the stratum corneum barrier function in diseased skin, TEWL has been chosen as the functional parameter. Such studies have demonstrated that in a number of skin diseases, the barrier function is impaired, as reviewed by Tupker *et al* [27]. The results of the present investigation confirm earlier findings of other investigators showing an increase of TEWL in lamellar ichthyosis patients [1–3,28]. Because the quality of the stratum corneum barrier is most likely dependent on the stratum corneum lipid composition and structure, studies examining these parameters may shed more light on the mechanism leading to the abnormality of the barrier function in diseased skin. The structural organization of human stratum corneum lipids in diseased skin has been studied only in a few cases using transmission electron microscopy in combination with the ruthenium tetroxide (RuO_4) post-fixation technique [17,29]. In the present study, we examined the lipid structure using SAXD on directly scraped stratum cor-

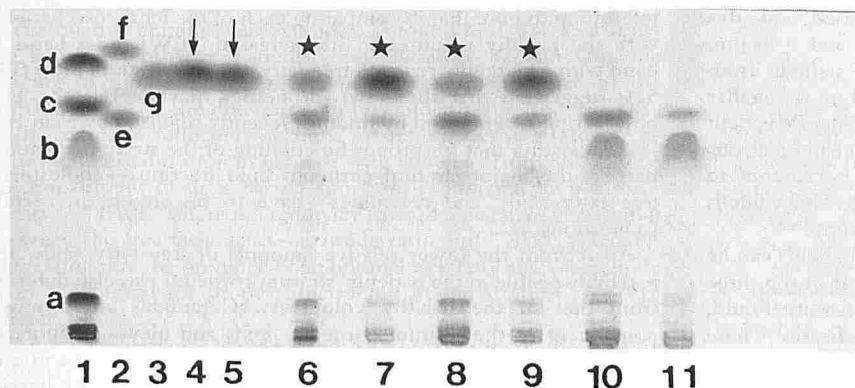


Figure 2. Identification of unknown component in lipids extracted from lamellar ichthyosis patients. Lipids collected by topical acetone/diethylether extraction were separated by HPTLC using the "lanosterol/lathosterol" development system. Figure shows mixture of lipid standards (lanes 1, 2), cetyl alcohol (lane 3), emollients used by the patients (lanes 4, 5), epidermal lipids of two patients (C and A) with lamellar ichthyosis (lanes 6, 7, 8, 9), and epidermal lipids of healthy volunteer 5 (lanes 10, 11). Lanes 7, 9, 11, initial 5-min lipid extracts; lanes 6, 8, 10, subsequent 25-min lipid extracts. Note the presence of a lipid fraction in the emollients (arrows) and in the patients (stars). a, ceramides; b, free fatty acids; c, cholesterol; d, diglycerides; e, lathosterol; f, lanosterol; g, cetyl alcohol.

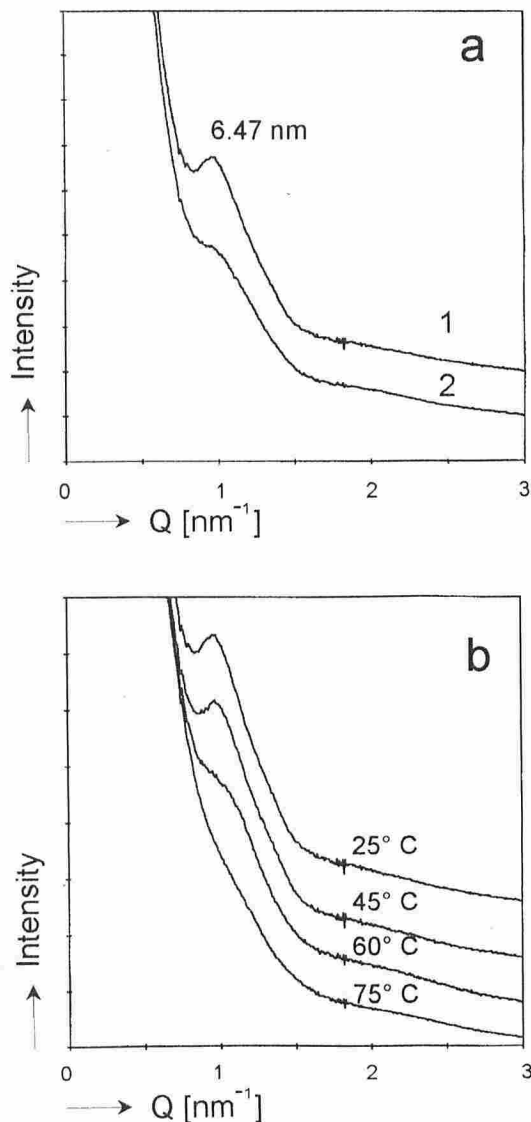


Figure 3. SAXD scattering curve of stratum corneum of healthy volunteers. The stratum corneum was collected by scraping, and SAXD measurements were performed a) at room temperature (healthy volunteers 1 and 2) and b) as a function of temperature (healthy volunteer 1).

neum. The results of the SAXD measurements revealed differences between patients and healthy volunteers in the organization of stratum corneum lipids. In ichthyotic stratum corneum, the single diffraction peak is located at significantly higher Q values in comparison with control stratum corneum. In diseased skin, this results in significantly smaller spacings (5.35, 5.38, and 5.90 nm, respectively) compared with the spacings found in samples from healthy volunteers (6.3 and 6.47 nm, respectively). A smaller lamellar spacing was also found by Ghadially *et al* [17] in one patient with lamellar ichthyosis. They found a spacing between electron-dense bands of 2.9 nm using transmission electron microscopy in combination with the RuO_4 post-fixation technique. This value is approximately half the value found in the present study.

An extra peak at $Q = 1.87/\text{nm}$ found in all three patients can be ascribed to crystalline cholesterol. In the lipid extracts of the three patients, relatively low amounts of free fatty acids were found, resulting in a decreased free fatty acid-to-cholesterol ratio. These findings strongly suggest that a certain free fatty acid content in stratum corneum is needed to facilitate solubilization of cholesterol

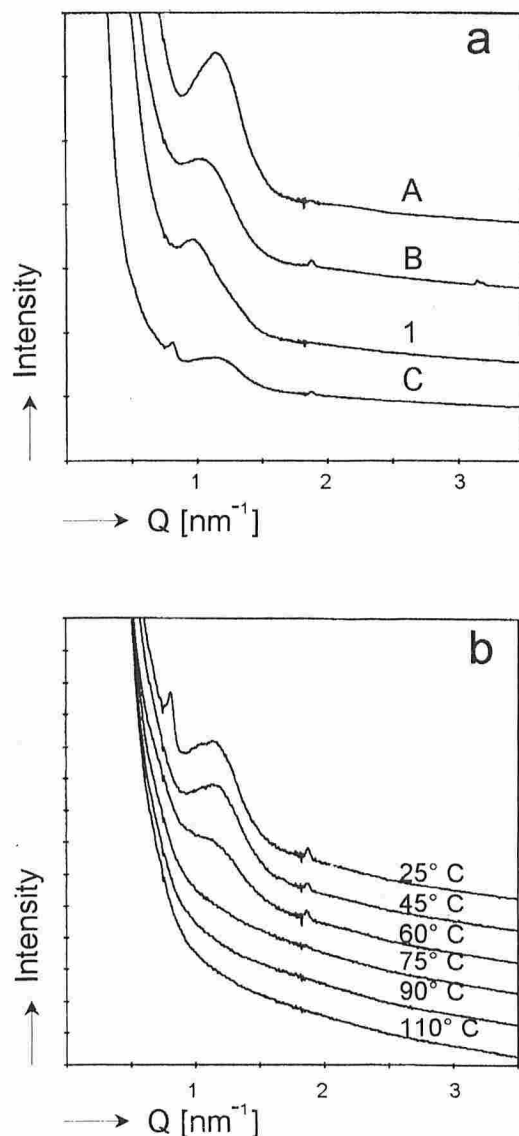


Figure 4. SAXD scattering curve of stratum corneum of patients with lamellar ichthyosis. The stratum corneum was collected by scraping, and SAXD measurements were performed a) at room temperature (the three patients and healthy volunteer 1) and b) as a function of temperature (patient C).

in the intercellular lipid mixture and imply that in these patients, this requirement is not met. The importance of the presence of a sufficient amount of free fatty acids for the solubilization of cholesterol has been demonstrated by Rehfeld *et al* [30]. It is interesting to note that in comparing each of the ichthyosis patients with the healthy volunteers, the increased TEWL goes hand in hand with a markedly reduced free fatty acid-cholesterol ratio (Fig 5a), suggesting that these two properties may be related. This observation is supported by findings recently reported by Man *et al* [31] indicating that for proper functioning of the stratum corneum barrier, the major stratum corneum lipid fractions—cholesterol, free fatty acids, and ceramides—have to be present in certain proportions.

Apart from the lower relative amounts of free fatty acids, the ceramide profile in the patients' stratum corneum was also different from that of the healthy volunteers. Ceramides are a major constituent of the stratum corneum lipids and play an important role in the barrier function of the epidermis [21,32]; the profile of the ceramides is probably an important determinant of the barrier

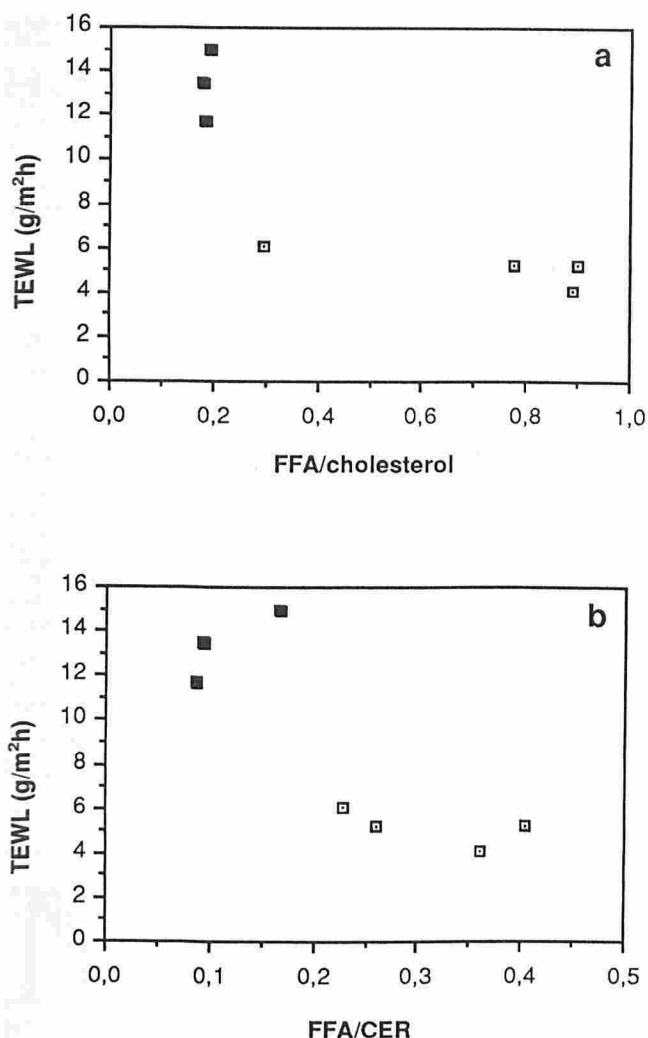


Figure 5. Relation between barrier function and stratum corneum lipid composition in patients with lamellar ichthyosis and healthy volunteers. Scatter plot of TEWL values with a) the weight ratio of the free fatty acids to cholesterol (FFA/cholesterol) and b) the weight ratio of free fatty acids to ceramides (FFA/CER) in three patients with lamellar ichthyosis (solid squares) and in four healthy volunteers (numbers 1, 4, 5, 6) (dotted squares). Lipids collected by topical acetone/diethylether extraction were separated by HPTLC.

function [33,34]. The ceramides differ in their structure of the sphingosine base (position and number of hydroxyl groups, double bonds, and the length of the long-chain base) and in the length and the structure of the alkyl chains [35]. The ceramide composition will therefore determine the packing of lipid lamellae. Differences in the relative amounts of ceramides, as found in the present study for lamellar ichthyosis patients, may therefore explain why the spacing of lipid lamellae in the patients is smaller than in the controls. Next to the differences in ceramide profile, the free fatty acids–ceramide ratio may account for differences in stratum corneum lipid organization in patients with lamellar ichthyosis and for the impairment of their barrier function, as assessed by measurements of TEWL. There is a tendency toward a negative correlation between the free fatty acids–ceramide ratio and TEWL (Fig 5b), suggesting that the presence of an optimal free fatty acids–ceramide ratio is required for proper functioning of the stratum corneum barrier.

Another difference between the SAXD patterns of the healthy volunteers and the patients was an additional peak at $Q = 0.82/\text{nm}$

found in patient C (corresponding d value 7.7 nm), which is normally not present. One can speculate that the appearance of this peak is due to the presence of an unknown fraction in stratum corneum lipid extracts as detected by HPTLC. This lipid fraction was also found in extracts of emollients used by these patients and is probably cetyl alcohol. Although this suggests that the cetyl alcohol is exogenous, endogenous sources (e.g., sebaceous glands) cannot be excluded.

For a clear understanding of the results obtained with SAXD on stratum corneum scrapings, it is necessary to put the methodology in perspective by comparing it with previous studies, largely based on the use of trypsinized stratum corneum [26]. In the trypsinized stratum corneum, the lipids are present in two lamellar phases with repeat distances of approximately 6.4 and 13.4 nm. Scattering curves from scrapings of healthy stratum corneum yield only one diffraction peak (6.5 nm spacing). These differences can be ascribed to several causes, including differences in the isolation procedure. We will address this issue in a future study. Although the information obtained with the scraping procedure differs from that obtained after trypsinization, this difference is expressed similarly in both healthy and ichthyotic samples, implicating reproducibility and therefore permitting comparison of the SAXD data collected from the two groups studied.

The present study shows that differences exist in the barrier function, composition, and organization of intercellular stratum corneum lipids between patients with lamellar ichthyosis and healthy volunteers. In each of the ichthyosis patients, TEWL was significantly elevated over the normal level observed in the healthy volunteers. Typically extracted stratum corneum lipids are composed significantly differently between ichthyotic and healthy skin samples regarding the relative amounts of various ceramides, free fatty acid–ceramide ratio, and free fatty acid–cholesterol ratio. The organization of the intercellular lipids in the scraped ichthyotic stratum corneum samples was markedly different from that of the normal samples based on the SAXD observations: Not only was the repeat distance of the bilayers significantly smaller, but also an additional peak, most likely representing crystalline cholesterol, showed up in the diffractograms of all patients.

It is possible that other factors also contribute to the barrier impairment found in patients with lamellar ichthyosis. Recent data suggest that abnormalities in cornified envelope formation occur in some patients with autosomal recessive lamellar ichthyosis [36]. In two of the three patients (A and C), deficiency in transglutaminase activity has been observed using immunohistochemical techniques [37]; this may arise from a functional deficiency of membrane-bound transglutaminase [38]. In addition, Huber *et al* [39] demonstrated defects in the gene that encodes for the enzyme transglutaminase in three families, in which five patients had lamellar ichthyosis (including patients A and C). These data suggest that not only lipids, but also structural proteins, are involved in the proper functioning of the stratum corneum barrier.

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